Symmetric Snapback Primers for Scanning and Genotyping of the Cystic Fibrosis Transmembrane Conductance Regulator Gene

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BACKGROUND: High-resolution melting of PCR products is an efficient and analytically sensitive method to scan for sequence variation, but detected variants must still be identified. Snapback primer genotyping uses a 5’ primer tail complementary to its own extension product to genotype the resulting hairpin via melting. If the 2 methods were combined to analyze the same PCR product, the residual sequencing burden could be reduced or even eliminated.

METHODS: The 27 exons and neighboring splice sites of the CFTR [cystic fibrosis transmembrane conductance regulator (ATP-binding cassette sub-family C, member 7)] gene were amplified by the PCR in 39 fragments. Primers included snapback tails for genotyping 7 common variants and the 23 CFTR mutations recommended for screening by the American College of Medical Genetics. After symmetric PCR, the amplicons were analyzed by high-resolution melting to scan for variants. Then, a 5-fold excess of H2O was added to each reaction to produce intramolecular hairpins for snapback genotyping by melting. Each melting step required <10 min. Of the 133 DNA samples analyzed, 51 were from CFTR patient samples or cell lines.

RESULTS: As expected, the analytical sensitivity of heterozygote detection in blinded studies was 100%. Snapback genotyping reduced the need for sequencing from 7.9% to 0.5% of PCR products; only 1 amplicon every 5 patients required sequencing to identify non-anticipated rare variants. We identified 2 previously unreported variants: c.3945A>G and c.4243–5C>T.

CONCLUSIONS: CFTR analysis by sequential scanning and genotyping with snapback primers is a good match for targeted clinical genetics, for which high analytical accuracy and rapid turnaround times are important. © 2013 American Association for Clinical Chemistry

High-resolution melting of PCR products is a simple and popular method for detecting and genotyping sequence variants (1–3). Scanning for genetic variants by high-resolution melting typically reduces sequencing needs by about 90% (4). Most coding and splice site sequences are wild type, and scanning quickly eliminates them from further consideration. The need for additional analysis depends on the frequency of sequence variants, which varies widely among genes. For example, sequence changes in the CYBB4 (cytochrome b-245, beta polypeptide) gene are uncommon, and less than 1% of its exons require further analysis (5), whereas 10%–20% of BRCA1 (breast cancer 1, early onset) amplicons may contain variants (6). Even when scanning conditions are carefully designed to optimize analytical specificity, polymorphisms that are not of clinical interest are found in about 5% of CFTR [cystic fibrosis transmembrane conductance regulator (ATP-binding cassette sub-family C, member 7)] amplicons (7, 8) and in 7% of BRCA1 and BRCA2 (breast cancer 2, early onset) amplicons (4, 9). Although scanning is an inexpensive, simple approach with an analytical sensitivity approaching 100% (1–3), its advantage over comprehensive sequencing depends strongly on minimizing subsequent analyses.

Several methods have been developed to minimize the need for sequencing after scanning. Common variants can be genotyped by methods that use the same reagents and instruments as for scanning. For example, the use of unlabeled probes (10) has reduced by 3-fold...
the sequencing burden after *BRCA1* and *BRCA2* scanning (11). Similarly, small-amplicon genotyping (12) and scanning can be performed in parallel to identify common polymorphisms (13). Typically, genotyping assays are performed after, or in parallel to, scanning assays that use different PCRs, although simultaneous scanning and genotyping with unlabeled probes is possible (14). For example, *CFTR* F508del homozygotes are difficult to detect by small-amplicon melting (15) but are easily genotyped with an unlabeled probe included in the scanning PCR (14). Simultaneous scanning and genotyping with unlabeled probes, however, requires asymmetric PCR, typically requires more PCR cycles, and is usually more difficult to optimize than symmetric PCR. Furthermore, splitting the signal between scanning and genotyping also lowers the dynamic range of each analysis and compromises scanning sensitivity.

Snapback genotyping uses an unlabeled probe attached to a primer’s 5′ end that is complementary to its own extension product (16). One strand is usually overproduced by asymmetric PCR to allow intramolecular hairpin formation and genotyping using the excess strand. In contrast, symmetric PCR with snapback primers leads to intermolecular hybridization of complementary full-length strands for scanning and not for genotyping. By diluting the scanned product with water, however, intramolecular hairpin formation is favored, and snapback genotyping can be performed via a subsequent melting analysis. This approach allows sequential scanning and genotyping of the same amplification product, thereby providing both optimal small-amplicon scanning and genotyping with up to 2 snapback primers. To minimize the need for sequencing in this study, we used this approach with snapback hairpins to sequentially scan and genotype all *CFTR* exons for the 23 mutations and 7 common polymorphisms recommended by the American College of Medical Genetics (ACMG). 5

Materials and Methods

**DNA SAMPLES**

We purchased 28 lymphoblastoid cell cultures established by Epstein–Barr virus transformation of peripheral blood mononuclear cells (Coriell Institute for Medical Research; see Table 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol59/issue7). These cultures, predominantly from cystic fibrosis patients with known genotypes, were expanded under culture conditions recommended by Coriell. DNA was isolated by lysis, protease digestion, anion-exchange purification, and isopropyl alcohol precipitation (Blood and Cell Culture DNA Midi Kit; Qiagen).

Associated Regional and University Pathologists kindly provided 25 DNA samples enriched for *CFTR* mutations (see Table 2 in the online Data Supplement). These samples had previously been genotyped by a 32-mutation panel, which includes the ACMG mutations. An additional 80 DNA samples were kindly provided by Associated Regional and University Pathologists and BioFire Diagnostics (see Table 3 in the online Data Supplement). These 80 samples were enriched for variants in *ACADM* (acyl-CoA dehydrogenase, C-4 to C-12 straight chain), *CYP2C9* (cytochrome P450, family 2, subfamily C, polypeptide 9), *VKORC1* (vitamin K epoxide reductase complex, subunit 1), *F2* [coagulation factor II (thrombin)], *F5* [coagulation factor V (proaccelerin, labile factor)], and *MTTHFR* [methylene-tetrahydrofolate reductase (NAD(P)H)] but with unknown *CFTR* genotype. These 105 DNA samples were whole-genome amplified (GenomePlex®; Sigma-Aldrich) according to the manufacturer’s instructions.

All cell culture- and whole genome–amplified DNA samples were dissolved in 10 mmol/L Tris (pH 8.0) and 0.1 mmol/L EDTA and were adjusted to 25 ng/μL according to their absorbance at 260 nm (NanoDrop® spectrophotometer; Thermo Scientific), on the assumption that an *A*<sub>260</sub> value of 1.0 is equivalent to 50 ng/μL.

**PRIMERS AND NOMENCLATURE**

Previously described *CFTR* primers (8) were modified with 5′ tails to enable snapback genotyping of the 23 ACMG mutations (17), p.I148T, and 7 common benign variants. Because 5 ACMG mutations are in exon 12, we used 3 different sets of tailed primers for genotyping, with each set based on the previous primer pair (8). The 39 primer pairs used had PCR product lengths varying from 162 bp to 324 bp. We did not modify 17 of these primer pairs because they did not encompass any of the targeted variants. Of the remaining 22 primer pairs used for genotyping, 16 had only 1 of the primers modified with a snapback tail; the 6 other pairs had both primers modified with snapback tails. Primers were synthesized with standard phosphoramidite methods by the University of Utah Core Facility. Table 4 in the online Data Supplement shows all primer sequences, product lengths, and both the legacy nomenclature and the Human Genome Variation Society nomenclature for variants and exons. The latter nomenclature is used throughout this report. Variants considered not associated with disease are either common variants described in the literature (p.M470V,
c.869+11C>T, c.-8G>C, and p.R75Q) or synonymous variants. All other variants are considered known or possible disease-associated variants.

**PCR ANALYSES**

PCR was performed in 10-μL volumes that included 25 ng genomic DNA, 0.5 μmol/L forward and reverse primers, 50 mmol/L Tris (pH 8.3), 2 mmol/L MgCl₂, 500 ng/μL BSA, 0.5× LCGreen® Plus (BioFire Diagnostics), 0.4 U KlenTaq1™ (Ab Peptides) with 64 ng anti-Taq antibody (eEnzyme), and 200 μmol/L of each deoxyribonucleoside triphosphate. PCR was performed in 96-well plates on a C1000 thermal cycler (Bio-Rad Laboratories). Two drops of mineral oil (about 25 μL) were added to each well. The plate was then covered with sealing tape and centrifuged (Eppendorf) at 1200 g for 3 min to remove air bubbles. PCR was performed as follows: an initial denaturation at 95 °C for 3 min and 35 cycles of 95 °C for 20 s, 62 °C for 15 s, and 76 °C for 15 s. After the PCR, the plate was heated to 95 °C, cooled to 15 °C, and either analyzed immediately or stored at 4 °C.

**HIGH-RESOLUTION MELTING ANALYSIS FOR SCANNING AND GENOTYPING**

After centrifugation at 1200 g for 3 min, the sealing tape was removed, and scanning curves were acquired in <5 min on a LightScanner® (BioFire Diagnostics) from 70 °C to 95 °C at 0.1 °C/s. Scanning curves were analyzed as described previously, with exponential background removal, normalization, overlay, clustering, and difference display (8, 18). We subsequently added 50 μL H₂O to each well, applied sealing tape, denatured the samples at 95 °C, and then cooled the samples to room temperature. After spinning the plate at 1200 g for 3 min, we removed the tape and acquired genotyping curves in <10 min on the LightScanner from 35 °C to 92 °C at 0.1 °C/s. Genotyping curves were analyzed by exponential background removal and normalization, with display on negative-derivative plots.

**Results**

Snapback primers were used for sequential scanning and genotyping of CFTR by high-resolution melting. After symmetric PCR (Fig. 1, A and B), scanning curves were obtained (Fig. 1, C and D). Then, a 5-fold excess of H₂O was added to each sample to favor formation of intramolecular hairpins (Fig. 1E). Subsequent melting produced genotyping curves (Fig. 1F) for definitive identification of common mutations and benign variants.

When snapback primers were designed for the 23 ACMG-recommended mutations and the 7 most common benign polymorphisms, amplicon-sequencing needs were reduced from 7.9% with scanning alone to 0.5% with scanning and snapback genotyping (Table 1). All variants in 2 blinded studies comprising 53 DNA samples enriched for disease-associated variants were detected by scanning and >90% of disease alleles were genotyped with the snapback primers (see Tables 1 and 2 in the online Data Supplement). Of the 80 random samples not enriched for CFTR variants, 4 had ACMG variants, 7 had other potential disease-associated variants, and 7 had synonymous variants that were not typed by snapback genotyping, requiring a total of 16 sequencing reactions for complete identification (Table 1; see Table 3 in the online Data Supplement). For this random population, 81% of the individuals (65 of 80) required no sequencing, and 15% (12 of 80) required sequencing of only 1 amplicon. For the 3 studies combined, 97% of the benign variants were successfully genotyped with snapback primers. After scanning and genotyping, two-thirds of the variants that required sequencing were known mutations or potential disease-causing variants, and one-third were common benign or synonymous variants. The rare variants that required sequencing after scanning and genotyping are tabulated in Table 5 in the online Data Supplement. We identified 2 previously unreported variants: p.I11315M (c.3945A>G) in exon 24 and c.4243–5C>T near exon 27. Table 6 in the online Data Supplement provides variant frequencies (excluding the 23 ACMG mutations) from this study and from our prior scanning study (8).

Twenty-five of the 28 CFTR snapback primers targeted only a single variant, with results similar to those presented in Fig. 1. The remaining 3 snapback primers targeted 2 variants by hybridizing over both loci. In exon 12, two nearby variants (p.R551D and p.R553X) were genotyped with 1 snapback primer, because all alleles had unique stabilities (Fig. 2). Similarly, p.F508del and p.I507del were both genotyped with 1 snapback primer, in combination with an opposing snapback primer that targeted M470V (Fig. 3). Distant variants in exons 3, 4, 8, 12, and 22 were also genotyped by using 2 snapback primers with hairpins of different stabilities that allowed all allele melting temperatures (Tms) to be separated. Another method for genotyping separated loci is to design a bulge into the snapback hairpin, as demonstrated for exon 23 for variants p.W1282X and c.3870A>G (Fig. 4).

As Fig. 1 suggests, complementary hairpins are formed after symmetric PCR of snapback primers. Fig. 5 shows in more detail the consequences of multiple hairpins with respect to melting analysis. Synthetic oligonucleotides were used to isolate the hairpins present when a common exon 15 variant, c.2562T>G, was genotyped. Although the Tms of the complementary wild-type hairpins are close, the stabilities of the vari-
Fig. 1. Sequential scanning and genotyping with snapback primers.

(A), A snapback primer includes a 5’ tail (diagonal crosshatch) that is the reverse complement of part of its extension product. It is capped with a 2-bp sequence that interrupts the reverse complementation (solid black). (B), Symmetric amplification with equal concentrations of primers produces a PCR product that includes the complementary tail sequences. Arrows indicate the 3’ end of each strand. Intermolecular full-duplex hybridization is favored at the end of the PCR because of the high ionic strength and strand concentrations. (C), High-resolution amplicon-melting analysis of CFTR exon 27 with a snapback primer targeted around the common variant, c.4389G>A. After exponential background subtraction, normalization, and curve overlay, most curves of the 174-bp duplex product cluster together (black), whereas some heterozygotes (presumably c.4389G>A) of different shape are identified (gray). (D), The same data as a difference plot. (E), After 5-fold dilution with water and denaturation, a reduced ionic strength and a lower strand concentration favor partial formation of intramolecular hairpins. The 2-bp cap prevents extension of the hairpin with the short 3’ end. (F), Repeat melting analysis reveals both intermolecular (amplicon) and intramolecular (hairpin) structures. Hairpin stability is greater for matched hairpins than for mismatched hairpins, allowing complete genotyping at c.4389.
ant hairpins are about 3 °C different because the mismatches are different. Any T_m difference between complementary hairpins widens the observed transitions of the homozygous genotypes and may limit the observed separation of alleles in the heterozygous genotype; however, complete separation of alleles is not necessary in heterozygote melting curves as long as the 3 genotype clusters are distinguishable by high-resolution melting.

Discussion

An interesting alternative to sequencing for research and clinical genetics is high-resolution melting (19). As a homogeneous method that requires only the PCR and fluorescence melting analysis, the advantages of simplicity, speed, and cost compared with conventional fluorescence sequencing are often cited (6, 11, 20–25). Massively parallel sequencing is a great discovery tool, but its throughput is mismatched with regard to targeted clinical diagnostics, in which a focused answer and a rapid turnaround time are more important than cost per base. In the case of single-gene disorders like cystic fibrosis, screening for multiple mutations has become standard rather than sequencing, partly because of cost and partly to avoid difficulties of interpreting the relevance of unknown variants. The analytical sensitivities and specificities of these methods are all very high. Diagnostic sensitivities and specificities vary widely with the extent of analysis, however. Focused genotyping has the lowest diagnostic sensitivity but the highest diagnostic specificity, sequencing (or scanning followed by sequencing) of exons and splice site junctions is intermediate, and massively parallel sequencing of the entire gene has the highest potential diagnostic sensitivity but also risks having the most false positives because it identifies more variants of unknown importance. Dealing with variants of unknown clinical relevance is problematic for the laboratory, physician, and patient.

Most sequence is wild type. For single-gene disorders, only 1 or 2 variants cause disease in any given individual, making the time and expense of whole-gene sequencing unnecessary and inefficient. Scanning methods either confirm the sequence as wild type or identify the locations of variation. High-resolution melting is now accepted as the best method of scanning because of its simplicity, speed, and low cost (1–3). Furthermore, most single-base changes can be genotyped via simple melting analysis of small amplicons (12), and all can be genotyped by melting unlabeled probes (10) or snapback primers (16). With snapback

### Table 1. Sequencing requirements after CFTR scanning and genotyping with snapback primers.

<table>
<thead>
<tr>
<th>Study</th>
<th>1&lt;sup&gt;a&lt;/sup&gt;</th>
<th>2&lt;sup&gt;b&lt;/sup&gt;</th>
<th>3&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Combined</th>
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<tbody>
<tr>
<td>No. of samples</td>
<td>28</td>
<td>25</td>
<td>80</td>
<td>133</td>
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<tr>
<td>No. of amplicons</td>
<td>1092</td>
<td>975</td>
<td>3120</td>
<td>5187</td>
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<td>Known mutations and potential disease-associated variants</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Genotyped by snapback primers</td>
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</tr>
<tr>
<td>Heterozygous, n</td>
<td>32</td>
<td>29</td>
<td>3</td>
<td>64</td>
</tr>
<tr>
<td>Homozygous, n</td>
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<td>2</td>
<td>0</td>
<td>6</td>
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<tr>
<td>Required sequencing, n (%)</td>
<td>5 (0.46)</td>
<td>2 (0.21)</td>
<td>11 (0.35)</td>
<td>18 (0.35)</td>
</tr>
<tr>
<td>Known benign and synonymous variants</td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>Genotyped by snapback primers</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heterozygous, n</td>
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<td>38</td>
<td>114</td>
<td>197</td>
</tr>
<tr>
<td>Homozygous, n</td>
<td>14</td>
<td>19</td>
<td>48</td>
<td>81</td>
</tr>
<tr>
<td>Required sequencing, n (%)</td>
<td>1 (0.09)</td>
<td>1 (0.10)</td>
<td>7 (0.22)</td>
<td>9 (0.17)</td>
</tr>
<tr>
<td>Amplicons requiring sequencing, n (%)</td>
<td>6 (0.55)</td>
<td>3 (0.31)</td>
<td>16&lt;sup&gt;d&lt;/sup&gt; (0.51)</td>
<td>25&lt;sup&gt;d&lt;/sup&gt; (0.48)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Blinded study of samples enriched for CFTR mutations purchased from the Coriell Institute for Medical Research.
<sup>b</sup> Blinded study of samples enriched for CFTR mutations, courtesy of Associated Regional and University Pathologists.
<sup>c</sup> Population study of samples retained for interesting genotypes of ACADM, coagulation (F2, F5, MTHFR) and warfarin susceptibility (VKORC1, CYP2C19), courtesy of Associated Regional and University Pathologists and BioFire Diagnostics.
<sup>d</sup> One sample had 2 rare variants (R668C and S686Y) that required sequencing but were in the same amplicon. Another sample had 1 mutation (p.W1282X) and 1 benign variant (c.3870A→G) in cis, both within a snapback hairpin that required sequencing. In both cases, only single sequencing reactions were required to identify 2 variants.
primers, scanning and genotyping can be combined into sequential steps of high-resolution melting that first scan for any variant and then genotype the common variants. This process leaves only residual rare variants that require genotyping via sequencing.

Sequential scanning and genotyping of CFTR with snapback primers directed to the 23 recommended ACMG mutations and 7 common polymorphisms reduced the frequency of unknown variants to 0.5% of all amplicons. With a >10-fold reduction in the need for sequencing, sequential scanning and genotyping has become an attractive alternative to targeted or massively parallel sequencing. When only 1 in 200 amplicons contain unknown variants, only 1 sequencing reaction is required for every 5 patients, a rate that can be decreased further by genotyping more variants. For example, by directing snapback primers to genotype an additional 2 synonymous variants (p.Y1424Y and p.T966T) and a syntenic pair of variants (p.G576A and p.R668C) that constitute a complex allele (26, 27), the sequencing required for the patients we have analyzed could be reduced by an additional factor of 2 (see Table 5 in the online Data Supplement). Although rare variants may differ between populations, subsequent targeting of additional variants requires only adding or modifying the snapback tails used for genotyping.

Snapback primer design is not difficult. In this study, 5 tails were added to previously designed primer pairs (8), with the variation kept near the middle and adjusting the tail length to obtain the desired hairpin Tm’s. Online tools are available to aid in snapback primer design (http://www.dna.utah.edu/usb/snap.php). Typically, tails are added to the primer that is closer to the variation to form the smaller hairpin

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**Fig. 2.** Scanning and genotyping of 2 mutations (p.G551D and p.R553X) in exon 12 of CFTR with 1 snapback primer. The mutations are separated by 4 bases in a 193-bp amplicon and are in the same single-nucleotide variant class, producing the same homoduplex and heteroduplex base pairs (12). Samples were run and analyzed in quadruplicate, including wild type (black), heterozygous c.1652G>A (blue), heterozygous c.1657C>T (green), and their compound heterozygote (red). (A), Normalized and overlaid amplicon-melting curves. (B), Difference plots. (C), Derivative melting curves of the snapback hairpin covering both mutations after dilution, denaturation, and cooling. The wild-type Tm is at 72 °C, p.G551D is at 64 °C, p.R553X is at 60 °C, and a compound heterozygote in trans shows a broad peak with a Tm of 62 °C. If both mutations were in cis, 1 wild-type allele (72 °C) and 1 allele doubly mismatched to the probe (<60 °C) would be expected in the melting curve.
loop. Smaller loops minimize the chance of additional secondary structure and increase hairpin $T_{m}$s so that shorter stems can be used (16). Bases mismatched to their target are added to the 5’ end of the tail to prevent extension from the mirror-image hairpin (Fig. 1).

If >1 variant within an amplicon needs to be genotyped, the snapback design depends on the position of the variants. Multiple variants within 15 bp can usually be genotyped with 1 snapback primer that cov-
Snapback Primer Analysis of CFTR Variants

Fig. 5. Multiple hairpins contribute to the observed melting curves from snapback genotyping after symmetric PCR. Hairpins with a 22-base loop, a 15-bp stem, and a 2-base terminal mismatch were synthesized to mimic the hairpins formed after amplification of CFTR exon 15 with a snapback primer targeted to genotype c.2562T>G (see Table 4 in the online Data Supplement). (A), Complementary hairpins 1 and 2, both of which were formed after symmetric amplification of wild-type DNA. The individual melting curves of the synthetic hairpins (gray lines) are close, but not identical, presumably because their loop sequences are different. When mixed together in a 1:1 proportion (as is produced after symmetric PCR of the wild-type sequence), the composite melting curve is between the 2 individual hairpins (black line). (B), Complementary hairpins 3 and 4, both of which were formed after symmetric amplification of the variant DNA. The C-T mismatch of hairpin 3 (black dots) is more destabilizing than the G-A mismatch of hairpin 4 (dashed line). This produces a separation in Tm between the synthetic hairpins of about 4 °C. When mixed in a 1:1 ratio, the composite melting curve, positioned between the individual hairpins, is the expected curve after amplification of the homozygous variant (thick black line). (C), The expected melting curves of all 3 genotypes, as constituted from the synthetic hairpins: the wild-type TT genotype as a mixture of hairpins 1 and 2, the variant GG genotype as a mixture of hairpins 3 and 4, and the heterozygous GT genotype as a 1:1:1:1 mixture of all 4 hairpins.

When variant loci are >15 bases apart, snapback tails can be added to both forward and reverse primers, and/or snapback primers that induce bulges in the template can be used (29). Fig. 4 shows an example of a bulge-inducing snapback primer, in which ACMG mutation p.W1282X is separated by 23 bases from the common variant, c.3870A>G. Deleting 10 bp between the 2 variants in the complementary snapback tail produces a hairpin that induces a 10-bp bulge in the template. This additional secondary structure keeps the hairpin Tm low enough to enable PCR and genotyping of widely separated variants. Positioning the variants nearer or farther away from hairpin loops or bulges allows variant Tm's to be separated for specific identification. Finally, if the number of variants is so great that
snapback genotyping becomes too complex, multiple parallel reactions can always be performed. Such was the case with the 5 ACMG mutations in exon 12, for which 3 reactions were performed to type 5 variants (see Table 4 in the online Data Supplement).

Genotyping with symmetric snapback primers after scanning, dilution with water, denaturation, and annealing to favor intramolecular hairpins is surprisingly robust. Indeed, it is easier to optimize and more often successful than the original asymmetric snapback primer genotyping that is limited to 1 primer (16). The combination of lower strand concentration and lower ionic strength of the diluted solution is critical to favor the formation of the genotyping hairpins over the full-length duplex product. After trying different solutions for dilution that varied in pH, ionic strength, dye, and DMSO concentration, a 5-fold to 10-fold dilution with water produced the best melting curves for genotyping. The 2-step sequential analysis maximizes both the scanning and genotyping signals, rather than dividing the fluorescence between scanning and genotyping, as in simultaneous scanning and genotyping with unlabeled probes (11, 14, 30).

Although we have found sequential scanning and genotyping with snapback primers an excellent way to decrease sequencing needs by >10-fold after scanning, there are some disadvantages. The intermediate step of dilution with water breaks the ideal closed-tube system and exposes the amplicon to the environment; however, this single in-tube dilution poses much less risk of contamination than the multiple steps required for sequencing. The dilution does lower the $T_m$ of both hairpin and amplicon, thus making $T_m$ predictions challenging. Rare homozygotes that are not genotyped with the snapback primers may not be detected unless mixing is performed (3). For example, a rare homozygous mutation (perhaps arising from a consanguineous marriage) that is not one of the 23 genotyped ACMG mutations would have a 5%–25% chance of being missed (28), depending on the instrument’s resolution. Finally, this method will not detect hemizygous whole-exon deletions or duplications, a property common to all PCR-based techniques.

Gene scanning and genotyping with snapback primers requires only 2 primers, symmetric PCR with a saturation dye, and high-resolution melting. No nucleotide modifications or probes are required. The quality of the resulting scan is the same as for standard high-resolution melting and superior to that after asymmetric PCR. There are no additional reagent costs for genotyping, and the method’s accuracy is enhanced by magnification of the hairpin (genotyping) signal over the amplicon (scanning). Each melting step requires <10 min. Sequential snapback scanning and genotyping of CFTR can reduce sequencing needs to <1 in 200 amplicons; similar rates should be obtainable for most genes.

With continued advances in massively parallel sequencing, will scanning/genotyping methods such as those we have presented become obsolete? It is hard to argue for whole-genome or whole-exome sequencing for a single-gene disorder, even at a vanishingly small cost per base. Hybridization-based targeted enrichment or long-range PCR of the entire gene (e.g., 20 overlapping fragments of 10 kb each) could be performed, but that requires subsequent library preparation with the addition of linkers and bar codes, as well as quality/quantity assessment for each sample before multiplex sequencing. Each step has a fixed cost and requires time. More direct would be the addition of bar codes and linkers during PCRs of 30–40 small amplicons bracketing the exons (31). Neglecting any quality/quantity assessment before emulsion PCR and all of the bioinformatics analyses, we estimate that the fixed cost per run after PCR (with a 10-Mb chip to capacity by analyzing 16 patients at 50× redundancy) might be as low as $350 (or $22 per patient) in today’s currency and take 1–2 days. Although this cost could be reduced by additional batching and using higher-capacity chips, the number of samples required per run would benefit only the highest-volume laboratories. In comparison, the fixed cost of the scanning/genotyping approach beyond that of the PCR is only the cost of the dye (<$2/sample), and the analysis time is about 30 min. The total time for scanning/genotyping (including DNA isolation, PCR, and 2 melting analyses) is about 4 h (5).

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References